Thioredoxin treatment increases digestibility and lowers allergenicity of milk

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Background: By resisting digestion in the stomach, the major bovine milk allergen, β-lactoglobulin, is believed to act as a transporter of vitamin A and retinol to the intestines. β-Lactoglobulin has 2 intramolecular disulfide bonds that may be responsible for its allergic effects.
Objective: This study was carried out to assess the importance of disulfide bonds to the allergenicity and digestibility of β-lactoglobulin.
Methods: β-Lactoglobulin was subjected to reduction by the ubiquitous protein thioredoxin, which was itself reduced by the reduced form of nicotinamide adenine dinucleotide phosphate by means of nicotinamide adenine dinucleotide phosphate-thioredoxin reductase. Digestibility was measured with a simulated gastric fluid; results were analyzed by SDS-PAGE. Allergenicity was assessed with an inbred colony of high IgE–producing dogs sensitized to milk.
Results: As found for other proteins with intramolecular disulfide bonds, β-lactoglobulin was reduced specifically by the thioredoxin system. After reduction of one or both of its disulfide bonds, β-lactoglobulin became strikingly sensitive to pepsin and lost allergenicity as determined by skin test responses and gastrointestinal symptoms in the dog model.
Conclusion: The results provide new evidence that thioredoxin can be applied to enhance digestibility and lower allergenicity of food proteins. (J Allergy Clin Immunol 1999;103:690-7.)

Key words: Thioredoxin, milk allergy, NADP-thioredoxin reductase, β-lactoglobulin allergenicity, β-lactoglobulin digestibility, milk allergy alleviation, milk allergy mitigation, thioredoxin and allergy alleviation/mitigation, dog model

Abbreviations used

BLG:	β-Lactoglobulin
DTT:	Dithiothreitol
mBBr:	Monobromobimane
NADP:	Nicotinamide adenine dinucleotide phosphate
NADPH:	Reduced nicotinamide adenine dinucleotide phosphate
NTR:	NADP-thioredoxin reductase
SGF:	Simulated gastric fluid

Food allergies continue to be an important human health problem, especially in children under age 3 years. Because of its massive consumption, bovine milk ranks among the top foods as a cause of childhood allergies. The long-known response to milk is caused by the proteins α-lactalbumin, serum albumin, caseins, and β-lactoglobulin (BLG).[1-3] The most prominent disulfide protein allergen, BLG, is believed to transport vitamin A and retinol from the mother to the calf.[4] Because of the presence of disulfide bonds, the question arises as to whether BLG is reduced by thioredoxin, as is the case for an array of animal and plant proteins containing intramolecular disulfide bonds.[5-10] It is also important to know whether reduction of BLG is accompanied by (1) an increase in digestibility, as found for various venoms,[9] seed trypsin,[5] and α-amylase inhibitors,[8] and (2) a decrease in allergenicity as observed with wheat gliadins and glutenins.[11]

Thioredoxin is a ubiquitous 12-kd protein with a highly conserved catalytically active disulfide group (Cys-Gly-Pro-Cys), which in seeds and animals is reduced by NADPH and the flavoenzyme, NADP-thioredoxin reductase (NTR).[12-14] We now report that, similar to the disulfide proteins studied earlier, BLG is reduced specifically by thioredoxin (see equations below). Furthermore, thioredoxin-reduced BLG showed both lower allergenicity and greatly enhanced digestibility, thereby opening the door to further testing of thioredoxin in the improvement of milk products.[15]

[1] $NADPH + H^+ + thioredoxin_{ox}\ (S\text{-}S) \rightarrow NADP^+ + thioredoxin_{red}\ (\text{-}SH\ HS\text{-})$

[2] $BLG_{ox}\ (S\text{-}S)$ allergenic nondigestible $+ thioredoxin_{red}\ (\text{-}HS\ SH\text{-}) \rightarrow BLG_{red}\ (\text{-}HS\ SH\text{-})$ less allergenic digestible $+ thioredoxin_{ox}\ (S\text{-}S)$

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METHODS

Animals: Background

The animals were derived from 6 highly pollen-reactive spaniels and retrievers selected for breeding in 1980 from a pool of 220 skin-tested bird dogs. Pups immunized at birth with grass and ragweed pollen extracts followed by live viral vaccines and pollen boosters made IgE antibodies by 4 months and experienced bronchospasm on inhalation of pollens at age 6 months. These dogs have been used over the past decade as an asthma model.[16,17] A similar immunization protocol was used with food allergens to which the dogs subsequently made IgE antibodies and experienced gastrointestinal signs on feeding challenges.[18] Clinically, only an occasional skin rash was observed, which is a shortcoming of this model of canine allergy in which dermatitis usually occurs. On the other hand, new evidence with wheat supports the validity of dogs as a model for food allergy in humans.[19] In a study with isolated protein fractions probed with sera from patients allergic to wheat, the order of strength of wheat allergens was gliadins greater than glutenins greater than albumins greater than globulins, as previously found for dogs.[11] Eosinophilic gastroenteritis was originally ruled out in the animals because of lack of peripheral blood eosinophilia, leukocytosis, normal absolute eosinophil counts in blood, and absence of blood in feces.[18,20]

Animals: Immunization schedule

From this inbred colony (6 generations) of highly allergic dogs, breeding resulted in 2 litters (12 pups), which were immunized with food extracts (commercial preparations of cow's milk, wheat, soybean, and beef, 1:10 or 1:20 wt/vol; Bayer).[18] At birth, pups were injected subcutaneously with 1 μg of each of these foods in 0.2 mL alum. At 3, 7, and 11 weeks, they received 0.5 mL each of distemper/hepatitis/parvovirus live vaccine subcutaneously followed 1 and 7 days after each injection with 1 μg of each food antigen in alum, which was also injected subcutaneously. Subsequently, they were boosted every 2 months with 1 μg of food allergen in alum. This colony of 12 high IgE–producing atopic dogs was maintained at the Animal Resources Service, University of California, Davis.

Allergen source

Raw cow's milk was obtained from the University of California, Davis. Pure BLG A and B and an 80% pure mixture of the 2 were from Sigma.

Chemicals and enzymes

Reagents and biochemicals, as well as thioredoxin, NTR, and glutathione reductase, were obtained from sources previously identified.[7] Porcine pepsin was from Sigma.

Skin tests

Procedures for skin tests to measure the type I hypersensitivity reaction have been described elsewhere.[11] In brief, Evans blue dye 0.5% (0.2 mL/kg) was injected intravenously 5 minutes before skin testing. Aliquots of 0.1 mL of whole cow's milk extract and pure β-lactoglobulin (reagent-grade, Calbiochem) were injected intradermally on ventral abdominal skin in half-log dilutions. Skin tests were read blindly by the same experienced observer, scoring 2 perpendicular diameters of each blue spot. Appropriate negative controls (diluted in physiologic buffer saline [10 mmol/L Na_2HPO_4, 1.8 mmol/L KH_2PO_4, 2.7 mmol/L KCl, and 137 mmol/L NaCl]) were included for each animal tested. Tests with thioredoxin, NTR, NADPH, and pepsin alone were consistently negative. To determine the effect of pepsin digestion on the allergenicity of the different forms of the proteins, the BLG or whole milk was either oxidized (untreated) or reduced with the NADP/thioredoxin system for 17 hours and then digested with simulated gastric fluid (SGF) (see below) before injection. A neutralized SGF control was consistently negative.

Feeding challenges

Reduction of 80% pure BLG (mixture of A and B forms) was performed in water (final volume, 100 mL). An aqueous mixture of 104 mg of NADPH, 1 mg of *Escherichia coli* thioredoxin, and 1 mg of *E coli* NTR was added to each gram of treated BLG. Incubations were carried out with shaking (125 rpm) at 37°C for 45 minutes. Samples were stored overnight at 4°C. The following day the treated (or untreated) BLG solution was mixed with a 12-oz can of D/D dog food (Hills) and fed to a dog. Control animals received dog food containing untreated BLG; unchallenged animals received dog food alone. The observations and recording of vomiting and number and quality of stools were made blindly by an experienced investigator or veterinary technician. Changes in the upper (vomiting) and lower (stool quality) gastrointestinal tract were scored as in Table I and subjected to the statistical methods described below.

Protein assay

Protein concentration was determined by the Bradford method with bovine gamma globulin as standard.[21] The concentration of pure BLG was determined by its absorbance at 278 nm by using a calculated molar extinction coefficient of 16,800.[22]

Protein modeling

A model of BLG structure, determined by Brownlow et al[23] at 1.8Å resolution, was provided by the Protein Data Bank, Brookhaven National Laboratory. A model of the protein with a single mutation C160S (partly reduced) and doubly mutated C160S-C106S (fully reduced) was built by the Swiss-Model program.[24] A 3-dimensional model of BLG was visualized by the RasMol program v2.6.

Protein reduction

Reduction of the disulfide bonds of proteins was performed as previously described[11] in a volume of 100 μL with one of the following: (1) the NADP/thioredoxin system, consisting of 5 μL of 25 mmol/L NADPH, 8 μL of 0.3 mg/mL *E coli* thioredoxin, and 7 μL of 0.3 mg/mL *E coli* NTR; or (2) the NADP/glutathione system, composed of 5 μL of 25 mmol/L NADPH, 10 μL of 30 mmol/L reduced glutathione, and 15 μL of 0.1 mg/mL glutathione reductase. Reactions were carried out in a 30 mmol/L physiologic-buffered saline solution containing either 10 μg pure target protein or 50 μg raw milk. The reaction mixtures were incubated at 4°C overnight or at 37°C and 55°C for 45 minutes. For complete reduction, samples were incubated in physiologic buffered saline containing 5 μL of 100 mmol/L dithiothreitol (DTT) and boiled for 5 minutes.

Pepsin assay

BLG, 640 μg, or milk, 1 mg protein, was incubated with or without the thioredoxin system at either 4°C (to yield the fully reduced form) or 55°C (to yield the partly reduced form), as described above. The BLG, 320 μg, or milk protein, 500 μg, was then treated in 200 μL of SGF (0.32% pepsin [wt/vol] and 30 mmol/L NaCl adjusted to pH 1.2 with HCl).[25] The reaction mixture was incubated at 37°C and stopped with a 0.375-fold volume of 160 mmol/L Na_2CO_3 to give neutral pH. The protein mixture was then subjected to SDS-PAGE (15% gels) and stained for protein with Coomassie blue as described below. As indicated, the allergenicity of the digested samples was determined by skin tests.

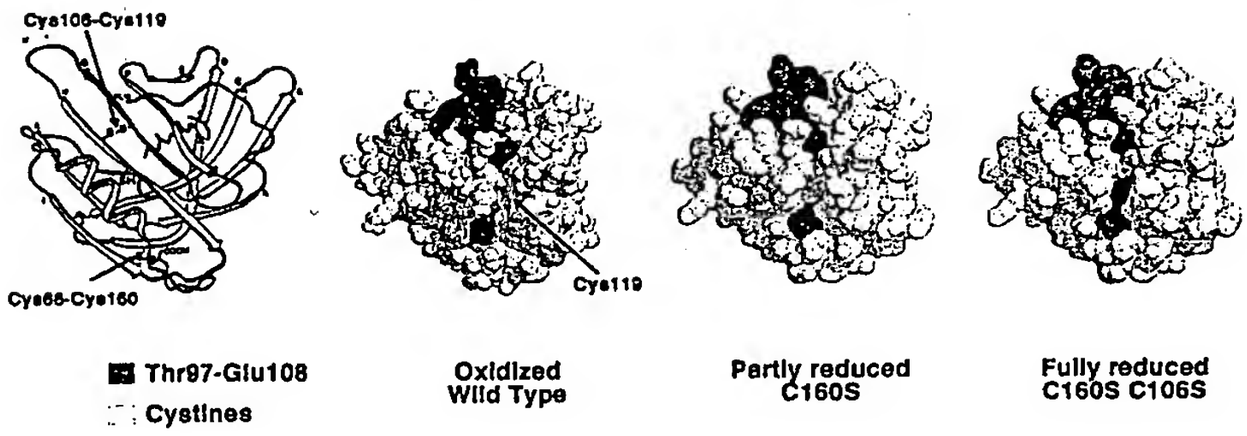


FIG 1. Localization of human antibody epitope of BLG *(left model)* and predicted effect of cystine mutagenesis on their accessibility *(right models)*. Epitope is identified in red. Partly and fully reduced BLG derivatives in the computer model on the right correspond, respectively, to mutagenesis (cys- > ser) of an exposed cys (C160S) and both an exposed (C160S) and hidden cys (C106S).[24,29]

Data analysis

The statistical significance of the thioredoxin-linked mitigation of the canine response to milk proteins determined by skin tests and changes in the upper and lower GI tract was determined by paired 1-tailed *t* tests. The null hypothesis assuming no difference in wheal area or gastrointestinal tract response induced by untreated versus thioredoxin-treated BLG or milk was tested against the alternative hypothesis that the treatment resulted in mitigation of allergic response. The paired 1-tailed tests were completed for each dilution series at the .05 level of significance on all sensitive dogs (df = 9).

Monobromobimane (mBBr) labeling and analysis of proteins

Sulfhydryl groups were visualized as their fluorescent mBBr derivatives.[26] Previous methods were used for SDS-PAGE analysis and quantitation.[11,26]

Sequence analyses

The different forms of BLG (oxidized and partly and fully reduced, 10 μg) were separated as mBBr derivatives by SDS-PAGE (10% to 20% acrylamide gradient, 1.5 mm thickness). An in-gel digestion method was used to obtain peptides containing Cys residues to allow structural characterization.[27] In brief, 1- to 2-mm pieces of the dried gel with the BLG bands were incubated with Lys-C endopeptidase (Wako). The resultant peptides were reduced with DTT and alkylated with iodoacetamide. This mixture was digested with trypsin, and newly formed peptides were purified on a C18 microbore column (1 mm × 15 cm, VYDAC) by using an Applied Biosystems 172 HPLC system. Purified peptides were sequenced by using either an ABI 477 or 470A sequencer. The disulfides of the fully oxidized protein correspond to Cys66-Cys160 (exposed disulfide) and Cys106-Cys119 (hidden disulfide). To identify the disulfide bonds involved in the partly and fully reduced forms, we isolated alkylated trypsin peptide(s) unique to each by comparing peptide maps of the oxidized form and 2 different reduced forms of BLG. The partly and fully reduced forms both showed the peptide indicator of the exposed disulfide: Leu149 to Ile162. The fully reduced protein also showed the peptide indicator of the hidden disulfide: Tyr102 to Val122.

RESULTS

BLG is known to contain 2 disulfides,[23,28] one clearly accessible on the surface and located close to the C-terminus (Cys66-Cys160) and the other close to the core located between 2 β-sheets (Cys106-Cys119 or possibly Cys106-Cys121) (Fig 1). Computer modeling revealed that when the exposed disulfide bond of BLG (Cys66-Cys160) was disrupted by site-directed mutagenesis,[24] a major human IgE epitope,[29] Thr97-Glu108, changed its structure and accessibility. Mutagenesis of the buried disulfide (Cys106-Cys119), which is a part of the epitope, led to little further change (Fig 1). A similar observation was made with a BLG model prepared with mouse IgG epitopes.[30] The experiments below were designed to test whether thioredoxin could reduce these disulfide bonds and if so whether a lowering of allergenicity accompanied reduction.

We first determined whether thioredoxin reduces BLG in milk by monitoring the redox state of the pure A and B forms after treatment with the NADP/thioredoxin system, consisting of NADPH, NTR, and thioredoxin. Samples were incubated with the system and then analyzed by the mBBr/SDS-PAGE procedure. Here the reduced (-SH) form of a target protein is derivatized with mBBr and, after separation by SDS-PAGE, appears as a fluorescent band when viewed in ultraviolet light.[5-10,11] As found earlier with a spectrum of proteins containing intramolecular disulfide bonds, the 2 forms of pure BLG (A and B) were actively reduced by thioredoxin.[5-10] When applied to whole milk, we observed that thioredoxin reduced not only BLG but also several other proteins, including α-lactalbumin, a subunit of lactose synthetase (Fig 2, *lane 2*). In conjunction with this work, we found that the enzyme lactose synthetase is inactive when its α-lactalbumin subunit was reduced by thioredoxin (data not shown). Similarly, when the isolated α-lactalbumin subunit was reduced by the NADP/thioredoxin system in the 37°C to 55°C temperature range, synthetase activity could not be reconstituted in the presence of the galactosyl transferase subunit.[31,32] The small band traveling in front of the major α- and β-casein components (24 kd) was identified as κ-casein. The other bands that became fluorescent after thioredoxin treatment have not been identified.

Temperature was found to affect the reduction of BLG significantly. When treated at 55°C (for 45 minutes), BLG was reduced (Fig 2, *lane 1* vs *2*), but its mobility in

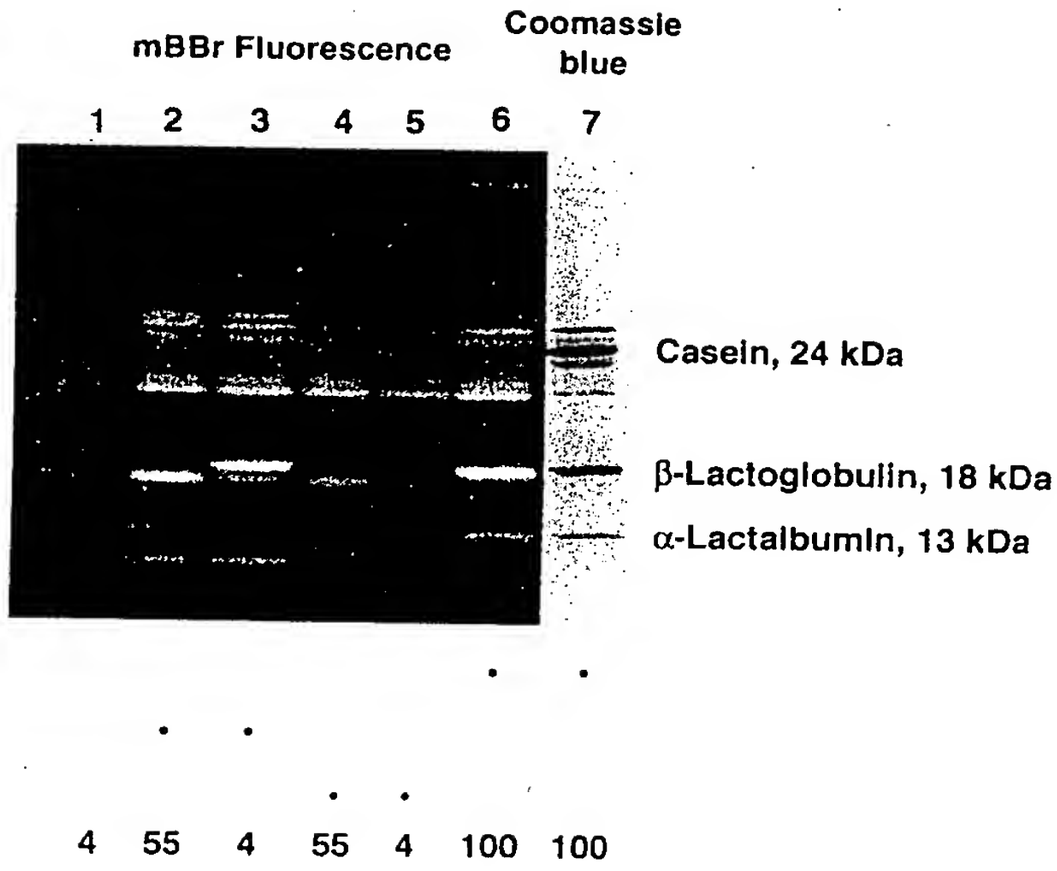


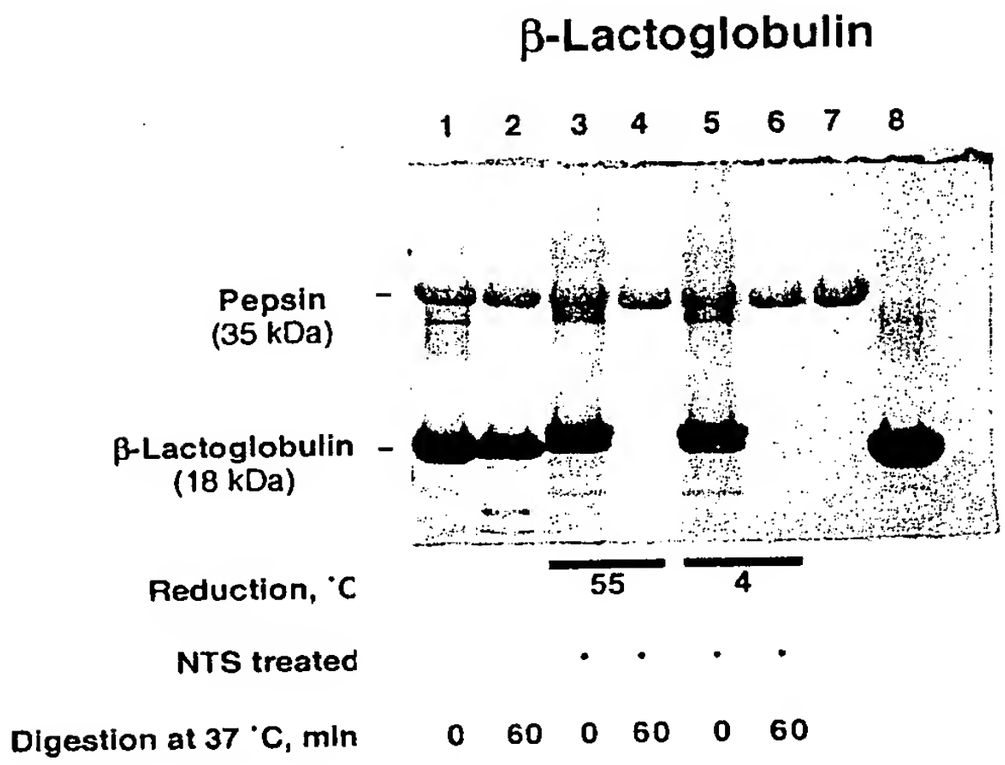
FIG 2. Reduction of milk proteins by the NADP-linked thioredoxin and glutathione systems determined by SDS-PAGE/mBBr procedure. After incubation as indicated, milk proteins were derivatized with mBBr, and fluorescence was visualized on SDS-PAGE. Raw milk (50 μg) was used in all samples. *Lane 1,* control incubated for 17 hours at 4°C with no addition; *lane 2,* NADP/thioredoxin system incubated for 45 minutes at 55°C; *lane 3,* NADP/thioredoxin system incubated for 17 hours at 4°C; *lane 4,* NADP/glutathione system incubated for 45 minutes at 55°C; *lane 5,* NADP/glutathione system incubated for 17 hours at 4°C; *lane 6,* DTT (5 mmol/L) incubated at 100°C for 5 minutes; and *lane 7,* same as lane 6 except stained with Coomassie blue. *NTS,* NADP/thioredoxin system; *NGS,* NADP-glutathione system.

the gel was only slightly changed. By contrast, when incubated at 4°C (for 17 hours), the mobility of the bulk of BLG decreased significantly, and a new form of the protein appeared (Fig 2, *lanes 3* and *6* vs *7*). An assessment of the extent of reduction by gel scanning revealed that BLG is partly reduced at 55°C and fully reduced at 4°C (see legend to Fig 2). Determination of the amino acid sequences of the tryptic peptides of partly and fully reduced BLG gave further information on the nature of the reduction.[23,28]

Sequence analysis confirmed that the exposed disulfide (Cys66-Cys160) seen in Fig 1 was reduced at 55°C (partly reduced form) and that this, as well as the hidden disulfide (Cys106-Cys119), were both reduced at 4°C (fully reduced form) (see legend to Fig 2). Differential reduction of BLG could also be achieved by altering pH. Thus reduction at pH 6.8 yielded only the partly reduced form, whereas a mixture of the partly and fully reduced forms was observed at pH 8.0 (both at 37°C) (data not shown). The results confirm that BLG becomes unstable at pH values above neutrality and undergoes conformational transitions at temperatures above 40°C.[33,34] We obtained the same reduction results without buffer (ie, by adding only the components of the NADP/thioredoxin system to raw milk). Under these conditions, the BLG in the milk treated at 4°C remained reduced for 2 days when retained at 4°C in air (data not shown).

As seen in Fig 2, the monothiol glutathione maintained in the reduced state by NADPH and glutathione reductase also reduced BLG, and to some extent other milk proteins, but much less effectively than thioredoxin (*lanes 4* and *5* vs *2* and *3*). The disulfide reductants, DTT and lipoic acid, were effective but only when combined with thioredoxin (data not shown).[6]

We have found that the trypsin sensitivity of small proteins containing intramolecular disulfide bonds (eg, trypsin and α-amylase inhibitors or venom neurotoxins) increases dramatically after reduction by thioredoxin.[5,8,9] BLG followed this same pattern: the thioredoxin-reduced protein was highly sensitive to trypsin digestion, whereas the oxidized protein was resistant (data not shown). Similar results were obtained with the pure BLG, as well as with milk subjected to SGF, as described by Astwood et al.[25] When separated by SDS-PAGE and stained with Coomassie blue, BLG was digested by pepsin, but only after reduction by thioredoxin (Fig 3, *left panel*). The difference in sensitivity was impressive; the oxidized BLG in milk resisted digestion for at least 60 minutes, whereas the thioredoxin-reduced form was digested within 60 seconds (Fig 3, *right panel*). Furthermore, the reduction of the more accessible disulfide bond (Cys66-Cys160) was sufficient. The partly (55°) and fully (4°) reduced forms of BLG showed no difference in pepsin sensitivity (Fig 3, *right upper* vs *middle* and *lower panels*). The glutathione-treated sample appeared to be insensitive to digestion (data not shown). BLG, either pure or in milk, was the only protein not visibly digested without reduction by thioredoxin (Fig 3, *right panel*). Previous investi-



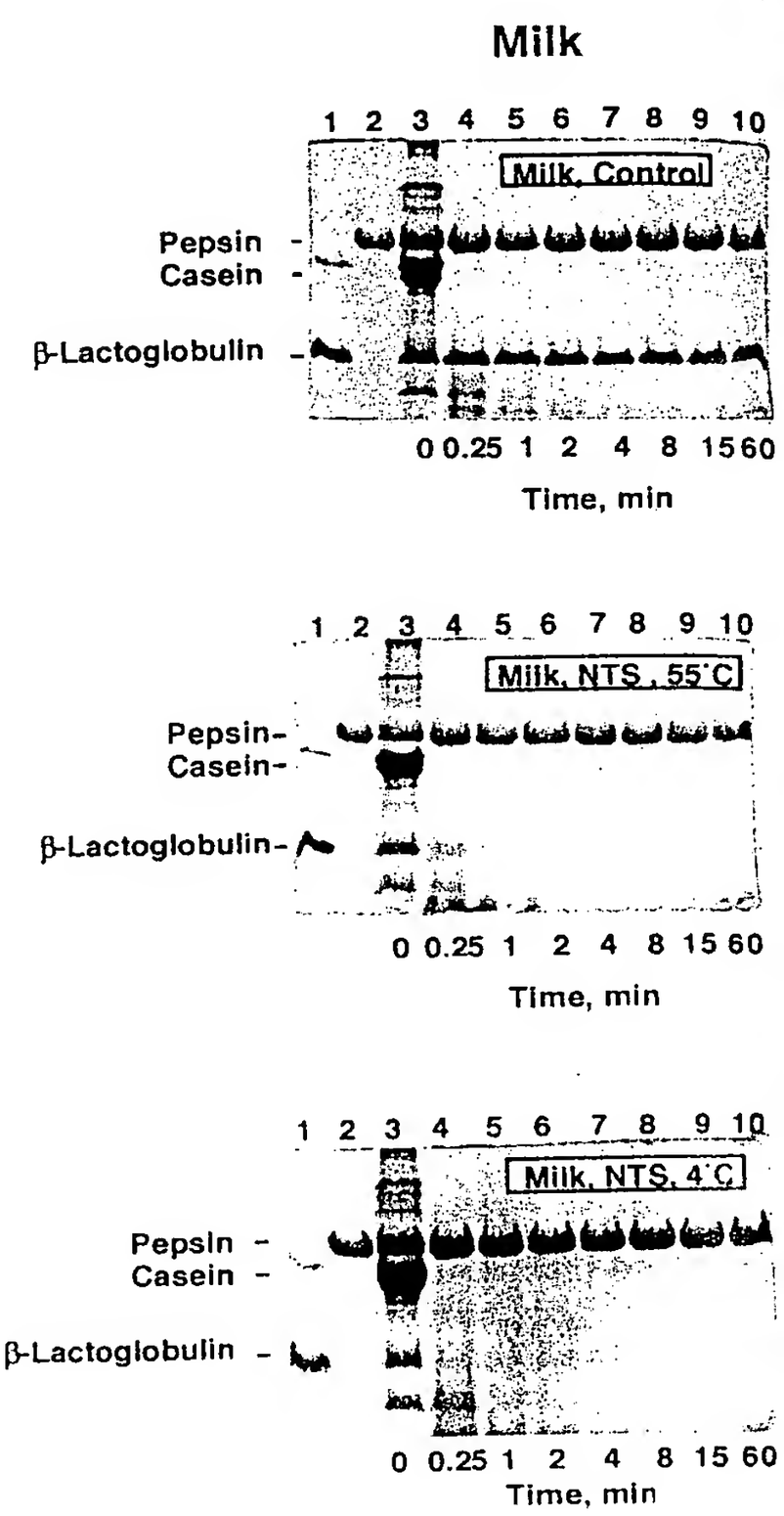


FIG 3. Effect of thioredoxin reduction on digestibility of bovine β-lactoglobulin *(left panel)* and milk *(right panel)* determined by SDS (15% gels)-PAGE/Coomassie blue procedure. All incubations (digestions) with SGF (see below) were at 37°C. *Left panel:* Samples 1 to 7 contained 13.5 μL of SGF. BLG 23 μg was added to samples 1 to 7. *Lane 1,* Untreated BLG was incubated for 0 minutes with SGF; *lane 2,* untreated BLG was incubated for 60 minutes with SGF; *lane 3,* BLG partly reduced by incubating for 45 minutes at 55°C with the NADP/thioredoxin system was then incubated for 0 minutes with SGF; *lane 4,* BLG partly reduced by incubating for 45 minutes at 55°C with the NADP/thioredoxin system was then incubated for 60 minutes with SGF; *lane 5,* BLG fully reduced by incubating for 17 hours at 4°C with the NADP/thioredoxin system was incubated for 0 minutes with SGF; *lane 6,* BLG fully reduced by incubating for 17 hours at 4°C with the NADP/thioredoxin system was then incubated for 60 minutes with SGF; *lane 7,* SGF alone; *lane 8,* BLG 32 μg alone. *Right panel:* Samples 2 to 10 contained 13.5 μL of neutralized SGF. Samples 3 to 10 contained 50 μg milk protein. Incubation time was varied as indicated. Milk control (BLG oxidized): *Lane 1,* BLG 5 μg standard; *lane 2,* neutralized SGF alone; *Lanes 3* to *10,* untreated milk was incubated for the indicated times with SGF. Milk NADP/thioredoxin system, 55°C (BLG partly reduced): *Lanes 3* to *10,* Milk partly reduced by incubating for 45 minutes at 55°C with the NADP/thioredoxin system was incubated for indicated times with SGF. Milk NADP/thioredoxin system, 4°C (BLG fully reduced): *Lanes 3* to *10,* Milk fully reduced by incubating for 17 hours at 4°C with the NADP/thioredoxin system was incubated for indicated times with SGF.

gators have also found the oxidized form of BLG and a number of other disulfide protein allergens to be resistant to proteases.[25,35]

In view of the above results, we sought to determine whether a mitigation of allergenicity accompanied the other changes associated with reduction by thioredoxin, as suggested by Fig 1. This question was pursued with sensitized dogs first by performing skin tests, as done earlier with wheat,[11] and second by feeding challenges.[18]

A skin test comparison of the allergenicity of the major proteins of milk (α-lactalbumin, BSA, BLG, and the α, β, and κ forms of casein) revealed that BLG is the

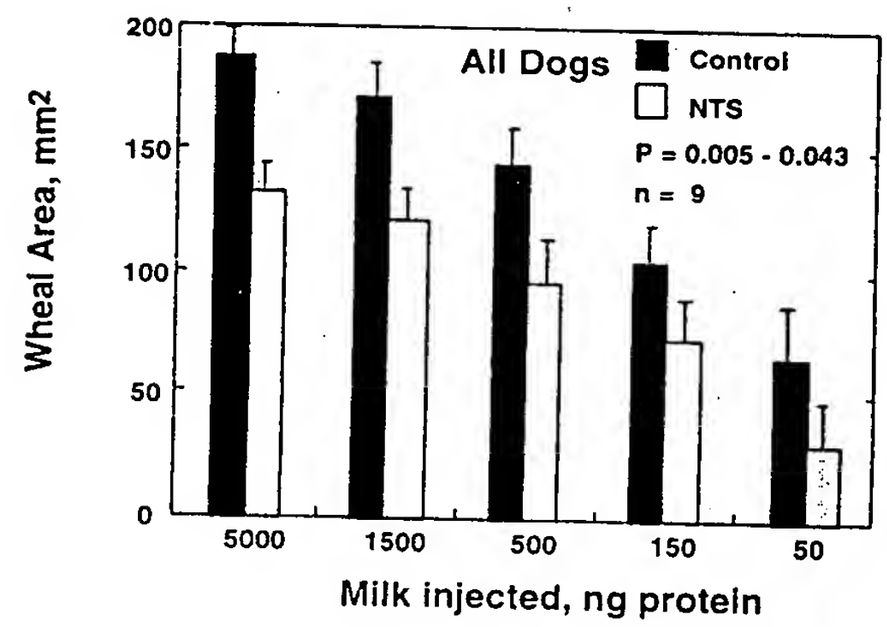
major allergen of milk, accounting for 80% of the total activity in the dog model. Of the other proteins, only BSA and, to a lesser extent, casein (β form) showed significant activity.

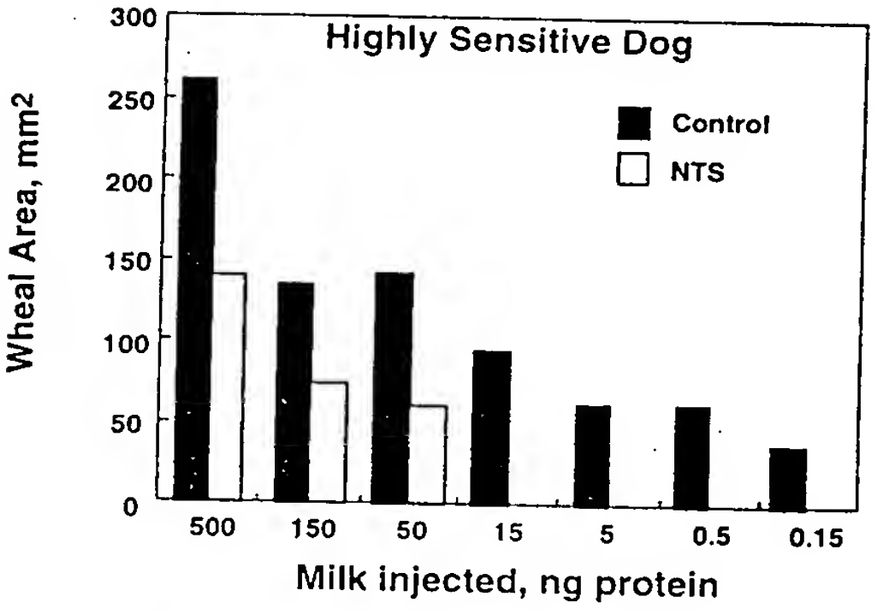
When treated with the thioredoxin system, both milk and BLG showed decreased ability to elicit an allergic response. Thus similar to our previous results with wheat,[11] the allergenicity of BLG and milk was decreased by a factor of 10 to 100, depending on the sensitivity of the dog tested. There was no difference between the pure A and B forms of BLG. Furthermore, as with wheat,[11] the results were statistically significant based on *t*-test analysis. Probability *(P)* values ranged from .043 to .005 as the allergen injected increased from 50 to 5000 ng in the 9 dogs tested (Fig 4, *top panel*). Among these dogs were highly and mildly sensitive animals that showed mitigation by thioredoxin treatment (Fig 4, *middle* and *lower panels*). There was no consistent difference among the animals in the allergenicity of the partly and fully reduced forms of BLG either pure or in milk (data not shown). This inconsistency likely reflects the fact that all dogs are not sensitized equally; they may recognize different epitopes.

The question arises as to the accessibility of the epitopes of BLG and other milk allergens when thioredoxin-treated samples are digested by pepsin in the SGF. Skin tests carried out with both BLG and milk revealed that peptic digestion nearly completely eliminated the allergenicity of both preparations. A wheal was observed in each case only with the highest level of protein injected (1000 ng). Furthermore, the activity of the digested samples was decreased to this marginal level irrespective of sensitivity of the individual dogs (8 animals tested).

An upset in the gastrointestinal tract leading to vomiting and diarrhea are clinical symptoms that accompany the ingestion of food allergens.[36] A minority of patients consistently show constipation rather than diarrhea.[37,38] The severity of these symptoms provides a clinical measure of the strength of allergens that complements skin testing. To obtain evidence on the gastrointestinal response, we supplemented the food fed to milk-sensitive dogs with BLG. The results revealed that the food containing reduced BLG significantly lessened the extent of vomiting relative to untreated BLG (Table I). Furthermore the response of individual dogs was reversed in most cases when their diets were switched (ie, from control to treated BLG and vice versa). Repeated trials in which dogs were each fed 1.25 or 2.5 g of the BLG indicated that, on average, about 70% of the gastric reflux disappeared on treating the BLG with thioredoxin. Here again the results were statistically significant. With diets containing 2.5 g BLG, the *P* value in the *t*-test analysis was .003. In all experiments the unchallenged dogs (fed dog food alone) consistently failed to vomit. Similarly, animals fed dog food containing thioredoxin (placebo control) also showed no clinical symptoms.

As shown in Table I, the extent of diarrhea or constipation, both symptoms of milk allergy, provided an independent measure of the ability of thioredoxin to mitigate





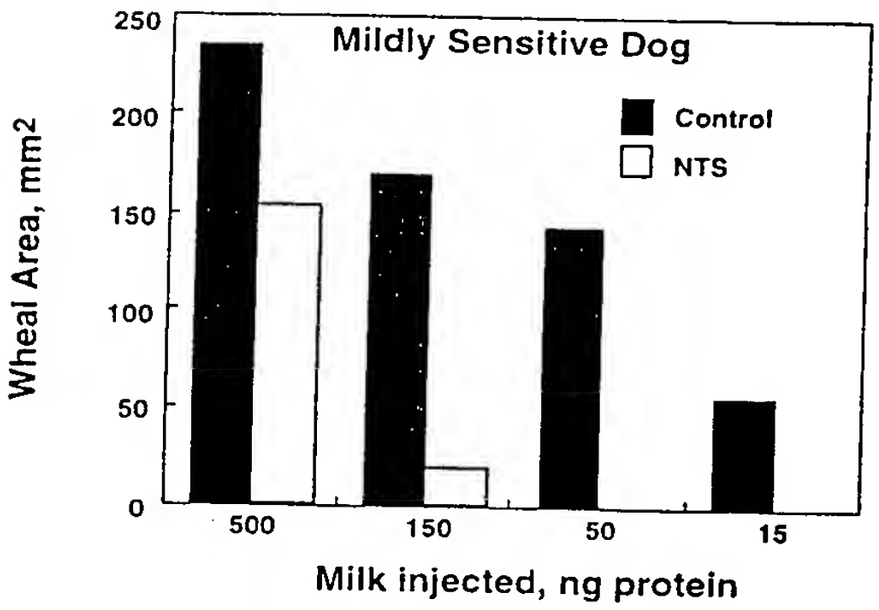


FIG 4. Thioredoxin-linked mitigation of milk allergic response determined by skin test responses from dogs of differing sensitivity. The type I hypersensitivity reaction was determined by the wheal area (mm^2) induced by 100 μL of intradermal injections of milk solution of known concentration either untreated (control) or reduced (with NADP/thioredoxin system) for 17 hours at 4°C. Physiologic-buffered saline and NADP/thioredoxin system controls were negative for each of the 9 dogs selected from the colony of 12. The remaining 3 dogs were inconsistent reactors. For the *top panel*, the *P* values, which were significant for each quantity of milk protein injected, were .043 (50 ng), .017 (150 ng), .041 (500 ng), .018 (1500 ng), and .005 (5000 ng). The *middle* and *lower panels* show the responses typical of highly and mildly sensitive dogs, respectively.

TABLE I. Allergic response of dogs alternately fed untreated (control) or thioredoxin-treated β-lactoglobulin

	Upper GI index: vomit scores*					Lower GI index: stool scores†			
	Experiment A		Experiment B			Experiment A		Experiment B	
Dog	Control	Treated	Control	Treated	Dog	Control	Treated	Control	Treated
6GCB3	7	—	—	0	5CBB6	1	—	—	0
6CGB7	6	—	—	3	6CGB1	0	—	—	0
6CGB1	—	0	5	—	6CGB4	2	—	—	2
6CGB4	—	6	9	—	6CGB6	0	—	—	1
6CGB6	—	0	8	—	6CGB8	3	—	—	0
					6CGB7	—	1	1	—
					6CGB9	—	1	2	—
					6GCB2	—	0	2	—
					6GCB3	—	0	4	—
					6GCB7	—	0	4	—

Upper gastrointestinal *(GI)* index response was measured by following the vomit response after feeding 2.5 g of the thioredoxin-treated or untreated BLG to dogs. Calculations are based on 2 separate feeding experiments in which the dogs were switched. Upper GI index quotations were as follows: no vomit = 0, limited vomit (<10 cm diameter) = 1, extensive vomit (>10 cm diameter) = 2, solid vomit = 1, liquid vomit = 2, liquid vomit with blood or bile = 3, delayed vomit = 1, and immediate vomit = 2 (maximum value = 9). Lower GI changes were followed on the challenge day and the following day. They were scored by the quality of the stools as follows: no change = 0, solid to semisolid or semisolid to liquid = 1, solid to liquid = 2, and constipation = 2. Scores reflect multiple vomits with numbers greater than 7.

*P = .003.

†P = .017.

the allergenic properties of BLG. In this case the effect of treatment with the NADP/thioredoxin system was monitored by changes in stool quality in the 2 days after food challenge. The intestinal disorder disappeared on average in 60% of the 10 dogs fed 0.25 or 0.625 g of thioredoxin-treated BLG. Again, the response of the individual dogs was generally reversed when their diets were switched. Analysis by the t test (P = .017) showed the trials to be statistically significant.

DISCUSSION

The present findings add a new dimension to recent work in which the principal allergens of wheat (proteins containing intramolecular disulfide bonds) caused a lower skin test response after reduction by thioredoxin.[11] In this study thioredoxin was found to act as a specific reductant for the intramolecular disulfide bonds of BLG, a major milk allergen. Depending on conditions, thioredoxin, reduced by NADPH and NTR, reduced either one or both of the disulfide bonds of BLG whether pure or in milk. The change in epitope distribution determined by skin tests and feeding challenges confirmed changes seen in structural models. The exposed disulfide (Cys66-Cys160) appeared to be more important than its buried counterpart (Cys106-Cys119) in maintaining the conformation responsible for allergenicity and resistance to digestion. This increase in sensitivity to pepsin (and trypsin) would likely lead to a rapid disappearance of ingested BLG in the gastrointestinal tract, thereby providing relief from the long-term effects of the allergen, notably edema and gastrointestinal upset.

As discussed earlier elsewhere,[11] others have previously observed the importance of intact disulfide bonds to the allergic response. In those studies the disulfide bonds were disrupted by a strong chemical agent, heat- or site-directed mutagenesis.[39-42] The novelty with thioredoxin is that mitigation is achieved by a mild and adaptable biochemical treatment consistent with its application to the improvement of food, in this case milk and, potentially, infant formulas. Thioredoxin treatment offers advantages over current improvement procedures in being independent of heat, proteolytic enzymes, and acid, agents that lower nutritional value and alter taste. Furthermore, the treated product is relatively stable, and on the basis of exploratory laboratory experiments with the dog model, alleviation of the allergic response is extensive. The disadvantages relate to the expense of ingredients (especially NADPH) and the need for a treatment system that accomplishes reduction without direct addition of the components of the system (NADPH, NTR, and thioredoxin). Experiments addressing these problems are currently in progress.

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